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Preliminary Amendment

Paper No. 15

UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of

Jan Vijg

Serial No.
[Continued Prosecution Application (CPA)
of parent application Serial No. 09/306,333
filed May 6, 1999]

Art Unit: 1655

Filed:

Examiner: Souaya, J.E.

For: BRCA 1 and bMLH I Gene Primer Sequences And Method For Testing

Hon. Commissioner of Patents
and Trademarks
Washington, DC 20231

Dear Sir:

This continued prosecution application has been necessitated in view of new issues raised in the current Office action (final rejection) of December 19, 2001, which replaced the earlier withdrawn final rejection.

The Office has explained (page 9 of said current Office action) that because "the Examiner erroneously indicated that the use of clamping and linking sequences constituted unexpected results", leading applicant to amend the claims accordingly, "the finality of the previous Office action has been withdrawn, and a new final rejection (the current Office action), addressing issues brought about by the amendment of the specification and claims after the first Office action", has been substituted.

In view of the subsequent heavy foreign and other business travel and related activities of both the applicant and counsel, it has taken all of the intervening months for applicant (now based in Texas) and counsel (in Boston) to locate and coordinate on further evidence, including mislaid publication and other materials, to satisfy the new Office requirement for a showing of "unexpected and improved results" attained by the invention over "obvious" variations of the Office-proposed combination of applicant's earlier teachings (referred to as "Vijg and Vijg II" by the Office).

Applicant has accordingly been required, in view of the finality of the Office action, to file this continuing application with the new evidence residing in his earlier misplaced published

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paper (a preliminary rough draft of parts of which served as the basis for counsel drafting the present application), entitled "A highly accurate, low cost test for BRCA 1 mutations" appearing in *J Med Genet* 1999; 36: 747-753, accepted for publication 21 June, 1999. A copy of this paper is herewith annexed.

The applicant makes this of record to make clear that applicant is not intentionally delaying the prosecution of this application by this continuing filing, but that applicant and counsel are diligently trying to address and satisfy the new requirements advanced by the Office to attain allowance of the application.

Please amend the parent application, accordingly, as follows:

Please delete from the substitute specification herein, "SEQ ID NOS 121 and 122" in the clamping sequence listings as erroneously inadvertently listed.

Please totally rewrite claim 10 as follows:

--10. (Rewritten) A method of detecting mutations in BRCA1 genes comprising providing PCR primers capable of amplifying the entire coding sequence of the BRCA1 genes; amplifying a test sample containing nucleotide sequences by long distance multiplex PCR with exon fragments numbered 10-11, 12-13, 14-17, 18-20, and 21-24, using primer sequences SEQ ID Nos. 37 and 38, 39 and 40, 41 and 42, 43 and 44, and 45 and 46, respectively, producing a first set of amplification products; subjecting this first set of amplification products to short distance multiplex PCR to produce a second set of amplification products with exon fragments numbered 11.1 F and R through 11.16 F and R, using primer sequence pairs SEQ ID Nos. 47 and 48 through 77 and 78, respectively, and exon fragments numbered 2 F and R through 10 F and R, and 12 F and R through 24 F and R, using primer sequence pairs SEQ ID Nos. 79 and 80 through 119 and 120, respectively, and with clamping and linking sequences therefor for effecting said short distance multiplex PCR; and subjecting the second set of amplification products to two-dimensional gel electrophoresis to produce a characteristic spot pattern for a specific mutation in the BRCA1 gene.--

Claim 11, line 2, please cancel "and hMLH1".

Please withdraw claims 7-9 which are to be presented in a divisional application pursuant to the requirement in the parent application for restriction.

Please amend claim 4 as follows:

-- 4. (Amended) Test kits for enabling BRCA1 gene testing comprising the primer pairs listed in Table 4 under "PRIMER SEQUENCES" column, mixed in about 20mM of Tris-HCl, 50mMKCl, 25pM of dNTP and 5% formamide.--

Please add the following claims:

- 12. The method of claim 10 wherein an eleventh exon fragment has been split 16 times to produce said exon fragments numbered 11.1 F and R through 11.16 F and R. --
- 13. The method of claim 10 wherein the primers for respective F and R exon fragments numbered 2-5, 11.1, 11.2, 11.4-11.6, 11.9, 11.10, 11.12, 11.14, 11.16, 12-18 and 22-24 are each clamped by a pair of clamping sequences. --
- 14. The method of claim 10 wherein the primers for respective F and R exon fragments numbered 6-10, 11.3, 11.7, 11.8, 11.11, 11.13, 11.15, and 19-21 are each clamped by a single clamping sequence. --

Remarks

The "new matter" reference in paragraph 2 of the current Office action and in the 35 U.S.C. 132 objection in paragraph 6, has now been cancelled, as required.

The indefiniteness of claims 4-6 and 10-11 has been obviated by the current amendments that clarify which primers with SEQ ID NOS 37-46 are paired with particular exon fragments, and which primers 47-120 are used with particular exon fragments, and which primers 47-120 are used with particular exon fragments and the clamping sequence attachments.

In claim 10, proper antecedent has now been provided.

It accordingly would appear that the 35 USC 112, second paragraph, rejection for indefiniteness should now be withdrawn.

The 35 U.S.C. 103 (a) Rejection

The claims have been rejected, in the absence of evidence of "unexpected and improved" results, as the "obvious" combination of the teachings of Vijg and Vijg II, modified "to provide a second GC clamp" as applied to detect mutations in the BRCA1 gene disclosed in both Liskay et al and Park et al; though the Office concedes that "Vijg does not teach testing gene sequences of the BRCA1 gene".

Unexpected And Improved Results

The before-mentioned Vijg et al paper submitted herewith reports that the particular specific now-claimed primers with specific sequence numbers paired with particular exon fragments, and as applied to the specific BRCA1 gene, not even suggested, let alone disclosed in any possible combination of the cited references, has indeed produced "unexpected" and "improved" results.

The relation of this paper to the present application (a draft of which, as before stated, served as the basis for drafting the current patent application) may be noted as follows.

The test procedures for the two-dimensional electrophoresis described for the BRCA1 at the bottom of page 4 and on page 5 of the original specification herein, are described on page 749 of said paper under the title "Two Dimensional Electrophoresis". The earlier PCR primer and amplification preparation for both "long-distance PCR" and "multiplex short PCR", including the "7-plex" PCR, etc., discussed on pages 3 and 4 of the original specification, are more fully detailed at the bottom of page 747 and on pages 748 and 749 of said paper.

"Figure 1" of the paper (page 748) and "Figure 2" on page 749 are identical respectively to the left-hand schematic chart and the right-hand photograph copy of original "Fig. 1B" of the original application. In view of the Office new comment (advanced for the first time during the prosecution of the application) that "the copy of these figures in the specification are unclear, especially the photographs" (bottom of page 9 of the current Office action), applicant respectfully requests permission to substitute copies of Figures 1 and 2 of the paper, which are clearer, for the original copy of Fig. 1B, and submits herewith a formal drawing therefor, further requesting permission for the use of the photographic copy since such shows much more detail than an attempt at a hand-drawn reproduction thereof would disclose.

Apart from the "highly accurate low-cost test for BRCA1 mutations" produced for the first time by the invention (and certainly not in the earlier cited work of Vijg), the abstract of the paper discloses that

"all 14 mutations (previously identified) were identified, as well as an additional five that had previously escaped detection".

The abstract also discloses that

"In addition to the 19 mutations, a total of 15 different polymorphic variants were scored, most of which were recurring."

It is submitted that none of these new and highly important results was either "obvious", or taught or even hinted at in any possible combination of the cited references, such that withdrawal of the 35 U.S.C. 103 (a) ground of rejection is appropriate and is accordingly respectfully requested.

Newly added dependent claims 12-14 depend from claim 10 more limitedly specifying clamping details and are thus also allowable.

Any costs incurred by this filing, including time extension fees in the parent application, petition for which extensions is hereby made, may be charged to deposit account number 18-1425 of the undersigned counsel.

Respectfully submitted,

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A highly accurate, low cost test for *BRCA1* mutations

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Abstract

The hereditary breast and ovarian cancer syndrome is associated with a high frequency of *BRCA1* mutations. However, the widespread use of *BRCA1* testing has been limited to date by three principal concerns: the fear of loss of health and life insurance, the uncertain clinical value of a positive test result, and the current lack of an inexpensive and sensitive screening test for *BRCA1* mutations. We have developed an inexpensive system for gene mutational scanning, based on a combination of extensive multiplex PCR amplification and two dimensional electrophoresis. The efficiency of this system, as a screening test for *BRCA1* mutations, was evaluated in a panel of 60 samples from high risk women, 14 of which contained a previously identified mutation in *BRCA1*. All 14 mutations were identified, as well as an additional five that had previously escaped detection. In addition to the 19 mutations, a total of 15 different polymorphic variants were scored, most of which were recurring. All were confirmed by nucleotide sequencing. The cost of screening per sample was calculated to be approximately US\$70 for the manual technique used in this study, and may be reduced to approximately US\$10 with the introduction of commercially available PCR robotics and fluorescent imaging. Implementation of this method of mutation screening in the research and clinical setting should permit rapid accrual of quantitative data on genotype-phenotype associations for the evaluation of diagnostic testing.

(J Med Genet 1999;36:747-753)

Keywords: genetic testing; two dimensional gene scanning (TDGS)

Breast cancer is a common disease with a life time risk in women of 10%. The risk increases substantially when one or more close relatives are affected. The first breast cancer susceptibility gene, *BRCA1*, was cloned in 1994.¹ To date, well over 300 distinct "high penetrance" mutations in this gene have been described by the Breast Cancer Information Core.² These confer a breast cancer risk of up to 90% by the age of 70.³ A second gene involved in hereditary breast cancer syndrome, *BRCA2*, was identified several months later,⁴ and at least one more gene remains to be found.⁵ Over 80% of families with two or more cases of premenopausal breast cancer and two or more cases of ovarian

cancer are believed to carry a *BRCA1* or *BRCA2* mutation.⁶ In addition, other genes may play a role as risk modifiers.⁷

Comprehensive evaluation of breast cancer genes could be used to provide women with information concerning the risk of developing cancer. Much recent work has been directed towards screening women from breast cancer families and other high risk populations to estimate the contribution of *BRCA1* mutations to the overall incidence of breast and ovarian cancers. On the basis of the results of these studies, attempts have been made to estimate the predictive value of a *BRCA1* mutation, that is, whether different mutations have a different risk.⁸ Information is now emerging that knowledge of *BRCA1* status is useful in preventive strategies. For example, oral contraceptive use reduces the risk of ovarian cancer by 50% in *BRCA1* carriers.⁹

One of the central themes in cancer risk assessment is the efficiency and accuracy of genetic screening methods. Currently, the ability to conduct large scale, population based studies is constrained by the lack of an accurate and inexpensive method for mutation detection. Nucleotide sequence based screening, which is currently accepted as the gold standard, is offered at US\$2400 for screening both *BRCA1* and *BRCA2* by Myriad Genetics (Salt Lake City, UT).

Here, we report on the evaluation of a practical test for *BRCA1* mutation detection, which is based on a combination of extensive multiplex PCR amplification, followed by two dimensional electrophoresis (TDGS, two dimensional gene scanning).^{10,11} The results of this study indicate a sensitivity for detecting mutations equal to that of sequencing, but at a much lower cost.

Methods

DNA SAMPLES

DNA samples from patients with known mutations in *BRCA1* were obtained from 60 patients with cancer and unaffected subjects. Mutations had originally been detected for 14 patients by sequence analysis of selected exons (exons 2 and 20) and by the protein truncation test of exon 11. No previous knowledge of mutation status of the panel was known when TDGS was applied.

PCR AMPLIFICATION

Multiplex PCR amplification was carried out in two steps. A 7-plex long distance PCR preamplification from genomic DNA provided the template for the amplification of the 37

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DGGE optimised target fragments in four multiplex groups (fig 1). Primers for long and short PCR were obtained from Genosys (primer sequences not shown because of lack of space but will be made available upon request). Long distance PCR reactions were performed as a 7-plex PCR, using 100 ng of total genomic DNA, 2.5 mmol/l $MgCl_2$, 400 $\mu\text{mol/l}$ of each dNTP, and 2.5 units of LA enzyme (LA PCR kit II, Takara) in a total volume of 25 μl in LA buffer. Primer concentrations were 0.125 $\mu\text{mol/l}$ (exons 10-11, 12-13,

18-20), 0.25 $\mu\text{mol/l}$ (exons 14-17, 21-24), and 0.5 $\mu\text{mol/l}$ (exons 1-3, 5-9). Amplification was carried out in a PTC-100 thermocycler (MJ Research) for 60 seconds at 94°C, after which the enzyme was added, followed by 32 cycles at 94°C for 15 seconds, 63°C for 60 seconds, 68°C for 12 minutes, and a final extension at 68°C for 12 minutes.

Multiplex short PCR was performed in four multiplex groups. Each group required 0.5 μl of long distance PCR products, 3 mmol/l $MgCl_2$, 250 $\mu\text{mol/l}$ of each dNTP, and 5 units of

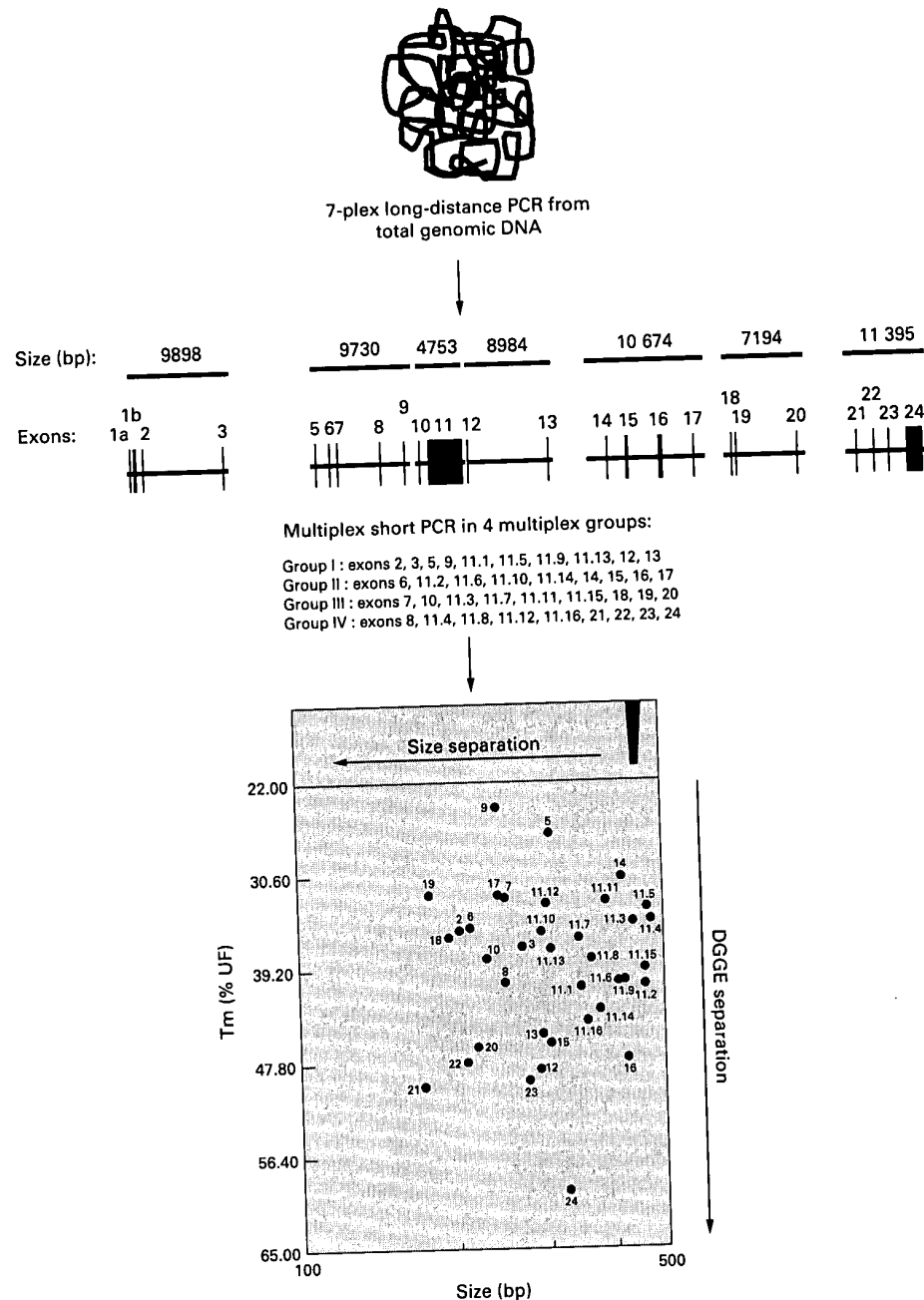


Figure 1 Schematic representation of TDGS as performed for BRCA1 mutation scanning. First, in a single multiplex long distance PCR, over-representation of the target sequences is obtained. Next, individual exons are amplified in four multiplex groups with exon 11 subdivided into 16 overlapping fragments. The products are combined and subjected to 2D electrophoresis, that is, fragments are first separated on the basis of size and subsequently further sorted according to base pair sequence in a denaturing gradient gel.

amplitaq Gold *Taq* polymerase (Perkin Elmer) in a total volume of 25 μ l in amplitaq Gold PCR buffer. Primer concentrations were 0.125 μ mol/l (exon 11.1), 0.25 μ mol/l (exons 9, 11.2, 11.5, 11.6, 11.7, 11.8, 11.12, 11.15, 12, 13, 17, 18, 19, 21, 22, and 23), 0.375 μ mol/l (exons 11.3, 11.9), 0.5 μ mol/l (exons 3, 6, 8, 10, 11.14, 14, 16, and 24), 0.75 μ mol/l (exons 2, 5, 7, 11.4, 11.10, 11.13, 15, and 20), 0.875 μ mol/l (11.11), and 1.25 μ mol/l (exon 11.16). Amplification was carried out in a PTC-100 thermocycler (MJ Research) at 95°C for 12 minutes,

followed by 10 cycles at 94°C for 40 seconds, 43°C for one minute minus 0.5°C per cycle, 72°C for one minute 30 seconds, 20 cycles at 94°C for 40 seconds, 40°C for one minute, 72°C for one minute 30 seconds with an increase of one second per cycle, and one cycle at 72°C for 10 minutes. Subsequently, PCR fragments were subjected to one round of complete denaturation and renaturation, that is, 98°C for 10 minutes, 55°C for 30 minutes, and 37°C for 30 minutes, to create heteroduplex molecules.

TWO DIMENSIONAL ELECTROPHORESIS

Five μ l of each multiplex group were combined and mixed with loading buffer (0.25% xylene cyanol, 0.25% bromophenol blue, 15% ficoll, and 100 mmol/l Na₂ EDTA). The mixture was directly loaded onto the top gel of an automatic 2D electrophoresis apparatus (CBS Scientific Co, Solana Beach, CA)¹², containing 10% polyacrylamide (acrylamide:bisacrylamide 37.5:1) and electrophoresed for four hours at 200 V in 1 \times TAE buffer at room temperature. Subsequently, fragments were further separated in the denaturing gradient part of the gel. The gradient used was 22-65% UF (100 UF=7 mol/l urea and 40% formamide) in 10% polyacrylamide and electrophoresis was for 16 hours at 100 V in 1 \times TAE buffer at 60°C. The gel was stained with a mixture of SYBR green I and II (Molecular Probes) and photographed under UV light using the Imager gel documentation system (Appigene).

NUCLEOTIDE SEQUENCING

Single fragments were amplified by PCR by using the same primers as used for TDGS, but without the GC clamps and with the exclusion of the heteroduplexing cycle. Twenty μ l of PCR product were run on a 2% low melting agarose gel and DNA was purified from the excised bands using the SurePure DNA extraction kit (Embi Tec, San Diego, CA). DNA concentrations were estimated by UV spectrophotometry and by comparison to a size marker in an agarose gel. Sequence reactions contained 90 ng of template, 2 pmol of primer, and 8 μ l of Terminator Ready Reaction Mix (Perkin Elmer). Cycling was carried out in a PTC-100 thermocycler for 25 cycles: 96°C for 10 seconds/ 50°C for five seconds/60°C for four minutes. Sequencing products were ethanol precipitated and run in an ABI Prism 310 Genetic Analyzer (Perkin Elmer). Sequencing results were compared to the published sequence of Smith *et al.*¹³

Results

Two dimensional gene scanning (TDGS) is based on a combination of extensive multiplex PCR and two dimensional electrophoresis. The latter comprises a size separation step followed by denaturing gradient gel electrophoresis (DGGE), and allows single base changes to be distinguished among multiple DNA fragments in parallel.¹⁰⁻¹¹ PCR amplification of the target sequences is carried out in two steps. First, all coding exons are amplified from genomic DNA by multiplex long distance PCR. Then,

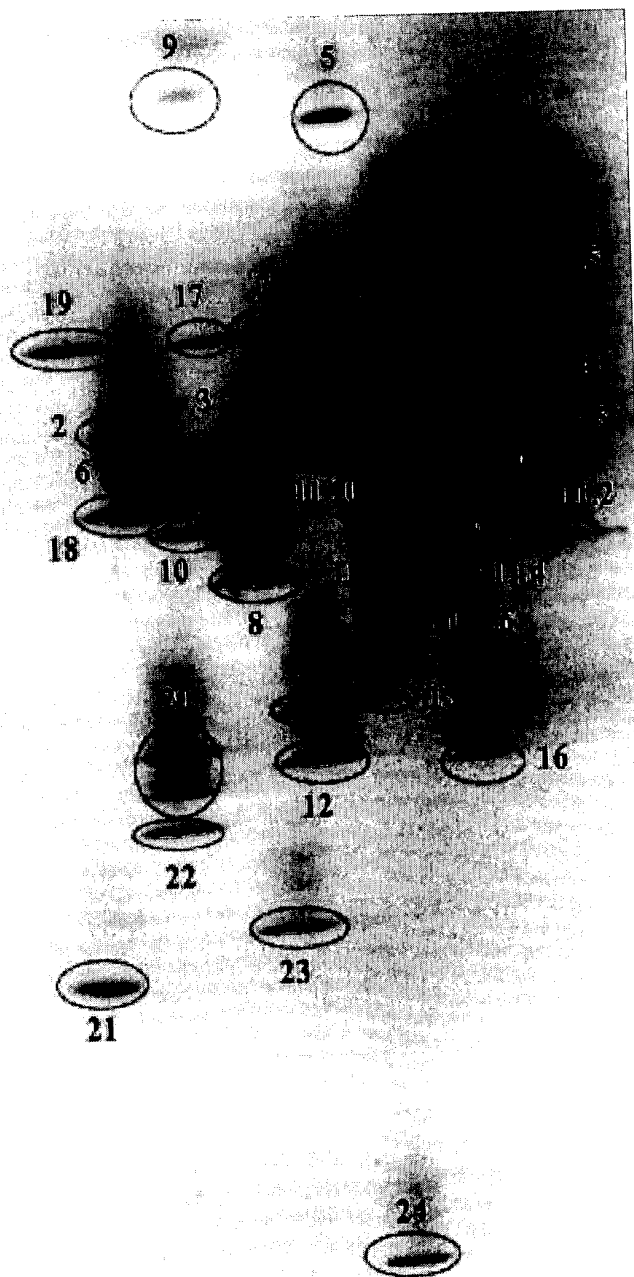


Figure 2 Empirical *BRCA1*-TDGS pattern of a control sample, containing polymorphisms in exons 3 and 20. Because of the heteroduplex step at the end of the PCR amplification, heterozygous variants are shown by the appearance of four rather than one spot: one homoduplex wild type (WT), one homoduplex mutant (MU), and two heteroduplex molecules (HE), each comprising one wild type and one mutant strand. The less stable heteroduplex molecules will melt earlier than the two homoduplex molecules (note the non-separated heteroduplexes in the exon 20 polymorphism).

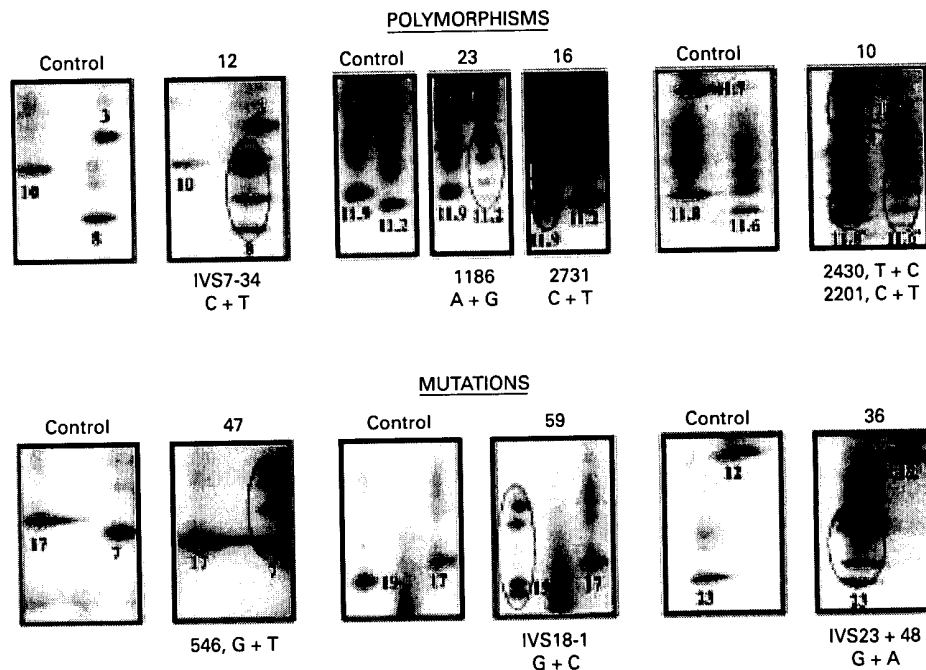


Figure 3 Examples of heterozygous polymorphisms and mutations in *BRCA1* as detected by TDGS. The top panels represent different frequently observed polymorphisms in exons 8, 11.2, 11.6, 11.7, 11.8, and 11.9 and the bottom panels represent different mutations in exons 7, 19, and 23 (tables 1 and 2).

the mixture of long fragments is used as the enriched template for the multiplex amplification of the relatively short (150 to 500 bp) DGGE optimised target fragments.¹⁴ PCR amplification of the *BRCA1* gene required a multiplex of seven long fragments serving as template for a total of 37 short PCR fragments. These fragments covered all coding exons of the gene (the non-coding exons 1a, 1b, and the non-coding part of exon 24 were excluded); 16 overlapping fragments were needed to cover the large exon 11 (fig 1). Two dimensional separation of the mixture of all *BRCA1* fragments simultaneously in a DGGE gel allowed detection of all mutational variants (fig 2). Heterozygous mutations or polymorphisms are detectable as four spots rather than one spot, representing the two homoduplex and the two heteroduplex variants. In some cases, the two heteroduplex variants are not well separated, resulting in the appearance of three instead of four spots. Examples of heterozygous mutations and polymorphisms are shown in fig 3.

To evaluate the sensitivity and specificity of this test, a panel of 60 DNA samples, previously screened for mutations in *BRCA1* using the protein truncation test, alone or in combination with partial nucleotide sequencing, was rescreened by TDGS (table 1). In 14 of these samples a *BRCA1* mutation had been identified. Twenty-seven samples were from family members of the mutation positive cases, but sequencing had found them to be non-carriers. A total of 19 samples were from familial cancer patients that had previously tested negative for exon 11 with the protein truncation test. Of the 14 previously identified mutations, 11 were in exon 11, and one mutation each was in exons 2, 8, and 20. All mutations

but one were predicted to lead to a truncated protein. Included were seven deletions of one to 40 nucleotides, four insertions of one nucleotide, and three base substitutions. TDGS was applied to the panel in a blinded fashion and all variants were detected (detection rate of 100%, table 1). No false negatives were found.

Among the 19 samples previously tested negative for exon 11 and rescreened by TDGS, none contained a truncating mutation in exon 11, which was expected. However, five variants were identified by TDGS. One of these was an unclassified variant in exon 11, but this was a single T to G nucleotide substitution, which leads to a Asn to Lys amino acid substitution. Two additional unclassified variants were detected. The first was a T to C at position 172, which leads to a Met to Thr substitution in exon 2. The second was a G to C in intron 18, which may influence splicing. Two pathogenic mutations were detected. The first was a G to T at nucleotide 546 in exon 7, which leads to a premature stop codon. The second was a single base pair substitution in intron 5, which leads to aberrant splicing and premature chain termination. These mutations have been described previously.^{2, 15}

In addition to the mutations and variants described above, a number of polymorphic variants were detected in the 60 samples. Many of these were identical, as indicated by their characteristic spot configurations. This was confirmed by individual analysis of each variant by 1D DGGE (results not shown). On the basis of different spot configurations, a total of 18 TDGS variants in 12 TDGS fragments were distinguished and confirmed by direct nucleotide sequencing (table 2). None of the variations was false positive. Comparison to the *BRCA1* sequence¹³ showed that these 18

Table 1 Mutational analysis of the *BRCA1* gene in 60 subjects by TDGS and sequence analysis

Sample No	Cancer*	Mutation in TDGS fragment	Exon	Nucleotide change	Additional heterozygous variants in TDGS fragments†
1	Br (bilat)	20	20	insC, 5382	11.6*, 11.7*, 11.8*, 11.9, 11.11*, 11.13, 11.14, 13, 16*
2	Br, Ov	2	2	delAG, 185	11.6*, 11.7*, 11.8*, 11.9, 11.11*, 11.13, 11.14, 13, 16*
3	Ov				
4					
5					
6		11.2/11.3	11	del40, 1294	11.6*, 11.7*, 11.8*, 11.9, 11.11*, 11.13, 11.14, 13, 16*
7					
8	Br, Ov	11.10	11	T→G, 3053	11.6*, 11.7*, 11.8*, 11.9, 11.11*, 11.13, 11.14, 13, 16*
9					
10	(Male)				11.6*, 11.7*, 11.8*, 11.9, 11.11*, 11.13, 11.14, 13, 16*
11					11.6*, 11.7*, 11.8*, 11.9, 11.11*, 11.13, 11.14, 13, 16*
12					11.6*, 11.7*, 11.8*, 11.9, 11.11*, 11.13, 11.14, 13, 16*
13					11.6*, 11.7*, 11.8*, 11.9, 11.11*, 11.13, 13, 16*
14	(Male)	11.14/11.15	11	insT, 3769	8
15					11.6*, 11.7*, 11.8*, 11.9, 11.11*, 11.13, 11.14, 13, 16*
16	Br	11.3/11.4	11	insA, 1559	8, 11.6*, 11.7*, 11.8*, 11.9, 11.11*, 11.13, 11.14, 13, 16*
17					11.6*, 11.7*, 11.8*, 11.9, 11.11*, 11.13, 11.14, 13, 16*
18	Br	8	8	delC, 633	8, 11.6*, 11.7*, 11.8*, 11.9, 11.11*, 11.13, 11.14, 13, 16*
19					8
20	Ov, Tong				11.6*, 11.7*, 11.8*, 11.9, 11.11*, 11.13, 11.14, 13, 16*
21	Mel				11.6*, 11.7*, 11.8*, 11.9, 11.11*, 11.13, 11.14, 13, 16*
22	(Male)				8, 11.2
23					11.9
24	Br	11.14/11.15	11	G→T, 3867	11.6*, 11.7*, 11.8*, 11.9, 11.11*, 11.13, 11.14, 13, 16*
25	Br				11.6*, 11.7*, 11.8*, 11.9, 11.11*, 11.13, 11.14, 13, 16*
26	Br				8, 11.6*, 11.7*, 11.8*, 11.9, 11.11*, 11.13, 11.14, 13, 16*
27	Br, Endo	11.4/11.5	11	C→T, 1806	8, 11.6*, 11.7*, 11.8*, 11.9, 11.11*, 11.13, 11.14, 13, 16*
28					8, 11.9
29	Ov	11.15	11	insT, 3879	
30					
31	Br	11.9	11	delAA, 2800	11.6*, 11.7*, 11.8*, 11.9, 11.11*, 11.13, 11.14, 13, 16*
32	Br, Ov	11.3	11	delAG, 1479	
33	Br	11.6	11	delA, 2190	
34	Ov	11.5/11.6	11	delGAAA, 2072	11.7*, 11.8*, 11.9, 11.11*, 11.13, 11.14, 13, 16*
35	Br				11.6*, 11.7*, 11.8*, 11.9, 11.11*, 11.13, 11.14, 13, 16*, 23
36	Br				11.6*, 11.7*, 11.8*, 11.9, 11.11*, 11.13, 11.14, 13, 16*
37	Br				8
38	Ov				8, 11.11*
39	Ov				
40	Ov				8, 11.6*, 11.7*, 11.8*, 11.9, 11.11*, 11.13, 11.14, 13, 16*
41	Ov				8
42	Br	6	Intron 5	T→G, IVS5-11	8, 11.6*, 11.7*, 11.8*, 11.9, 11.11*, 11.13, 11.14, 13, 16*
43	Cx, Br (bilat)				
44	Br				
45	Cx, Br, Ov				
46	Br				
47	Br (bilat)	7	7	G→T, 546	
48	Cx, Br				8, 11.2
49	Br				11.6*, 11.7*, 11.8*, 11.9, 11.11*, 11.13, 11.14, 13, 16*
50	Br				
51	(Male)				11.6*, 11.7*, 11.8*, 11.9, 11.11*, 11.13, 11.14, 13, 16*
52	Br				11.6*
53	Br				11.6*, 11.7*, 11.8*, 11.9, 11.11*, 11.13, 13, 16*
54	Ov	11.14/11.15	11	T→G, 3827	11.6*
55	Br, Ov				8, 11.6*, 11.7*, 11.8*, 11.9, 11.11*, 11.13, 11.14, 13, 16*
56	Ov	2	2	T→C, 172	8, 11.2
57	Br (male)				11.2
58	Br				8, 11.2, 11.6*, 11.7*, 11.8*, 11.9, 11.11*, 11.13, 11.14, 13, 16*
59	Br	19	Intron 18	G→C, IVS18-1	13, 16*
60	Br, Ov				

*Br = breast, Ov = ovarian, Tong = tongue, Mel = malignant melanoma, Endo = endometrial, Cx = cervix.

†Sequence analysis is depicted in table 2.

**See table 2.

different spot configurations comprised 15 different sequence variants (table 2).

Of the 15 variants confirmed by sequence analysis, 10 were known polymorphisms, three were unclassified variants, and two were novel variants not found in the BIC database (table 2). Two of the three unclassified variants occurred in subjects in whom a mutation in the gene had also been identified (samples 6 and 8, tables 1 and 2). The third unclassified variant was found in a patient with ovarian cancer for whom no *BRCA1* mutation had been identified (sample 39 in table 1). One of the two novel variants (seen in one of 60 subjects), an A to T transversion at position 3107 resulting in a Lys to Asn amino acid change, was found in the

same fragment (fragment 11.11), which already contained an unclassified variant. Neither nucleotide sequencing nor PTT had previously detected this variant in this patient with ovarian cancer (sample 39, tables 1 and 2). The second novel variant was also found in only one subject, in exon 23 (sample 36). Interestingly, this IVS23+48G/A occurred in a patient with breast cancer in which no other *BRCA1* mutation could be detected.

As mentioned above, the characteristic spot configuration of recurrent mutations and polymorphisms allows their identification once they have been sequenced. Fig 4 shows three different spot configurations for fragment 11.6 with some of the surrounding fragments as refer-

Table 2 Sequence analysis of polymorphic variants in *BRCA1* detected in the panel of 60 subjects

Exon	TDGS fragment	Nucleotide position*	Nucleotide change	Type of variant	Codon	Amino acid change	Type of polymorphism†
8	8	IVS7-34	C→T	Transition	—	—	Polymorphism
11	11.2	1186‡	A→G	Transition	356	Gln→Arg	Polymorphism
	11.6 ^a	2201‡	C→T	Transition	694	Ser→Ser	Polymorphism
	11.6 ^b	2196‡	G→A	Transition	693	Asp→Asn	Polymorphism
		2201‡	C→T	Transition	694	Ser→Ser	Polymorphism
		2196‡	G→A	Transition	693	Asp→Asn	Polymorphism
	11.6 ^c	2196‡	G→A	Transition	771	Leu→Leu	Polymorphism
	11.7 ^a = 11.8 ^a	2430‡	T→C	Transition	772	Val→Ala	Unclassified variant
	11.7 ^b = 11.8 ^b	2434	T→C	Transition	871	Pro→Leu	Polymorphism
	11.9	2731‡	C→T	Transition	1038	Glu→Gly	Polymorphism
	11.11 ^a	3232	A→G	Transition	996	Lys→Asn	Not reported
	11.11 ^b	3107	A→T	Transversion	1040	Ser→Asn	Unclassified variant
13		3238‡	G→A	Transition	1183	Lys→Arg	Polymorphism
	11.13 = 11.14	3667‡	A→G	Transition	1436	Ser→Ser	Polymorphism
	13	4427‡	T→C	Transition	1613	Ser→Gly	Polymorphism
16	16 ^a	4956‡	A→G	Transition	1652	Met→Ile	Unclassified variant
	16 ^b	5075	G→A	Transition	—	—	Not reported
23	23	IVS23+48	G→A	Transition	—	—	Not reported

*Nucleotide numbering starts at the first transcribed base according to GenBank entry U14680.

†Named according to BIC as of 10.2.99.

‡Common polymorphisms defining haplotypes (see also table 3).

Exon 11, Fragment 6

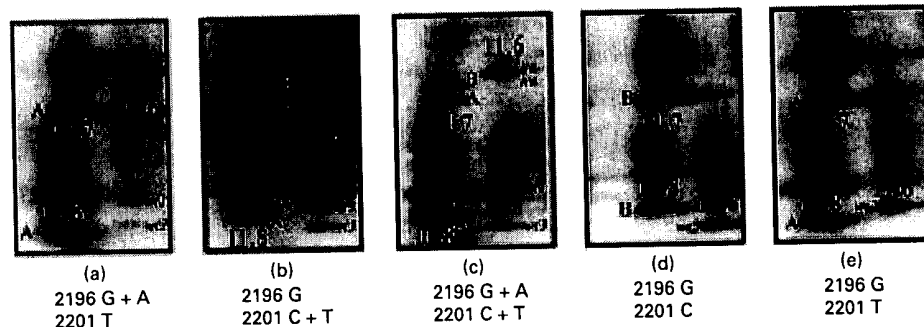


Figure 4 Examples of the effect of different variations in the same TDGS fragment. Panels a-c show three different patterns of heterozygosity in fragment 11.6, whereas panels d and e each show a different pattern of homozygosity.

ence points. Each of these three configurations represents a frequently observed heterozygous variant associated with a specific polymorphism. Sequence analysis indicated that two positions in this fragment, 2196 and 2201, are polymorphic. The fragment in fig 4a is heterozygous at position 2196 (G + A) and homozygous at position 2201 (T), the fragment in fig 4b is homozygous at position 2196 (G) and heterozygous at position 2201 (C + T), and the fragment in fig 4c is heterozygous at both positions. So far, for fragment 11.6, a total of three alleles were identified, that is, *wt1-3* (fig 4a-c).

The corresponding homozygous situations were more difficult to recognise from the 2D pattern solely by eye. The use of the surrounding spots as markers was complicated by the frequent occurrence of variations in these fragments as well (see, for example, the variation in fragments 11.7 and 11.8 in fig 4b and c). Nevertheless, the fragment 11.6 homozygous spots in fig 4d and e could be identified as alle-

les *wt3* and *wt2* respectively, which was confirmed by a subsequent 1D DGGE experiment (results not shown).

A recurrent pattern of identical spot configurations, which occurred in exon 8, seven of the fragments of exon 11, exon 13, and exon 16, was also noted. These variants represented polymorphisms that could be arranged into haplotypes (table 3). One of the benefits of parallel screening by TDGS is that each pattern shows all heterozygous variants in the entire gene.

Discussion

This study shows that *BRCA1* mutation screening by TDGS is an accurate method of mutation detection. Previous evaluations of TDGS tests for other large human disease genes support the claim that DGGE based tests are as accurate as nucleotide sequencing.¹⁶⁻¹⁸ It may be that heterozygous mutations are even easier to detect on the basis of the DGGE principle (that is, three or four spots rather than one) than by sequencing (that is, two overlapping peaks). TDGS shows all heterozygous variations in the entire gene, as illustrated by the high number of variants detected. A total of 34 mutations and variants were observed in a population of 60 subjects. This technique will enable epidemiologists to record all variations within the gene for the establishment of genotype-phenotype correlations.

Table 3 Polymorphisms defining haplotypes

Type	Exon 11						Exon 13	Exon 16	%*
	2196	2201	2430	2731	3232	3667	4427	4956	
A	G	C	T	C	A	A	T	A	63
B	G	T	C	T	G	G	C	G	28
C	A	T	C	T	G	G	C	G	7
D	G	C	T	T	A	A	T	A	2

*Occurrence in 126 chromosomes.

In TDGS, recurrent polymorphisms can be recognised based on their unique spot distribution pattern, but new variants must be sequenced to determine the exact nature of the observed variation. Theoretically, 300 single base substitutions are possible along a 100 base pair DNA fragment. However, so far, an average of only 14 variations has been observed per *BRCA1*-TDGS fragment (information derived from the BIC). Thus, the possibility that a mutation in a given fragment will show the exact same spot configuration as a known polymorphism is small. However, it has become clear that for a gene containing many polymorphic sites, unequivocal identification of homozygously occurring alleles is only possible by careful examination of the variant in the context of the surrounding spots, which can also represent polymorphic fragments. Increased standardisation and the application of automated image analysis systems containing information on the spot positions of all known variations should greatly facilitate spot identification for this purpose.

During the course of this study, attention was given to the actual costs of the test. It has previously been shown that the maximum throughput of TDGS for a typical large disease gene, for example, the retinoblastoma susceptibility gene *RBI*, is 60 tests per technician per week using manual procedures.¹⁹ The costs for *BRCA1* are higher, because of the need for four instead of only one multiplex groups, and was calculated to be about US\$70 per test. The most prominent cost components in the manual procedure involve labour and PCR enzymes. The costs of PCR primers were low since these were based on the total number of reactions possible with the lowest volume that could be ordered. It should be noted that had each fragment been separately amplified rather than coamplified the costs associated with enzymes and labour would have been significantly higher.

The costs of the assay can be dramatically reduced by automation. PCR robotics reduces both labour and reagent costs (smaller PCR volumes), while fluorescent imaging using dye labelled primers obviates the need to stain the gels. The costs per sample are then mainly determined by the primers that have to be labelled with a fluorophore, while labour and enzyme costs become vanishingly small. According to our calculations, these proposed automation steps will drive the costs down to about US\$10 per test, which is approximately one to two orders of magnitude lower than current sequencing protocols.¹⁹ This anticipated increase in cost-efficiency is based solely on commercially available instrumentation and not on technological improvement.

The TDGS test is ideal for large studies to establish the potential value of screening for mutations in *BRCA1* as an aid to breast cancer management. However, the hereditary breast cancer syndrome is genetically heterogeneous and other susceptibility genes have been identified, including *BRCA2*, *TP53*, *PTEN*, and, arguably, *ATM*.²⁰⁻²⁴ In order to use genetic

screening as an aid for risk assessment of breast cancer, a battery of genetic tests should be applied. TDGS is flexible since its design permits the rapid expansion of a test to include additional genes. TDGS tests, which screen multiple genes in a single assay, can and have been developed.¹⁰

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